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Report

Ajuba LIM Proteins Are Negative Regulators of the Hippo Signaling Pathway

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Summary

The mammalian Ajuba LIM proteins (Ajuba, LIMD1, and WTIP) are adaptor proteins that exhibit the potential to communicate cell adhesive events with nuclear responses to remodel epithelia [1, 2]. Determining their role in vivo, however, has been challenging due to overlapping tissue expression and functional redundancy. Thus, we turned to *Drosophila*, where a single gene, *CG11063* or *djub*, exists. *Drosophila* lacking the *djub* gene or depleted of dJub by RNA interference identify *djub* as an essential gene for development and a novel regulator of epithelial organ size as a component of the conserved Hippo (Hpo) pathway, which has been implicated in both tissue size control and cancer development [3–9]. *djub*-deficient tissues were small and had decreased cell numbers as a result of increased apoptosis and decreased proliferation, due to downregulation of DIAP1 and cyclin E. This phenocopies tissues deficient for Yorkie (Yki), the downstream target of the Hippo pathway. *djub* genetically interacts with the Hippo pathway, and epistasis suggests that *djub* lies downstream of *hpo*. In mammalian and *Drosophila* cells, Ajuba LIM proteins/dJub interact with LATS/Warts (Wts) and WW45/Sav to inhibit phosphorylation of YAP/Yki. This work describes a novel role for the Ajuba LIM proteins as negative regulators of the Hippo signaling pathway.

Results and Discussion

The *Drosophila* Ortholog of Mammalian Ajuba LIM Proteins, dJub, Regulates Organ Size

In *Drosophila*, there is a single ortholog of the mammalian Ajuba subfamily of LIM proteins encoded by the *CG11063* locus in the X chromosome [10]. CG11063 exhibits greater sequence similarity to the three mammalian Ajuba subfamily proteins than to dZyx, the *Drosophila* ortholog of the Ajuba-related zyxin subfamily of LIM proteins (see Figure S1A available online). We designate CG11063 as dJub (*Drosophila* Ajuba LIM proteins).

To determine the in vivo function (or functions) of *djub* in *Drosophila*, we generated two dJub RNA interference (RNAi) lines: *djub-RNAi 22.5* and *djub-RNAi 18.1* (Figure S1A). Ubiquitous expression of either transgene via *actin-GAL4* resulted in pharate lethality, suggesting that *djub* is an essential gene. Both RNAi constructs yielded similar phenotypes in all subsequent assays. Because *djub-RNAi 22.5* consistently induced stronger phenotypes, we refer to RNAi 22.5 with the phrase "dJub RNAi" henceforth.

Because Ajuba LIM proteins are abundant in mammalian epithelia [11] and have been implicated in epithelial functions [1, 2], we selectively depleted dJub in larval wing and eye imaginal disc epithelium. dJub RNAi expression in the wing decreased wing size to 65% of wild-type (Figures 1B and 1E). Western blot analysis revealed a 60% reduction of dJub protein level by RNAi (Figure 1H). The small-wing phenotype was due to decreased cell number, not cell size, and wing patterning appeared unaffected (Figures 1B and 1F). Similarly, expression of dJub RNAi in the pupal eye epithelium resulted in a 25% reduction in interommatidial cells, without disruption to ommatidial patterning (Figures 1J and 1K). These RNAi phenotypes were specific for dJub depletion, because overexpression of a wild-type (WT) *djub* transgene in dJub RNAi-expressing cells partially rescued both wing and eye phenotypes (Figures S1D and S1H). Furthermore, overexpression of human LIMD1 (most closely related to dJub) rescued the dJub RNAi wing phenotype (Figures S1G and S1H), suggesting that this function of Ajuba LIM proteins is conserved between *Drosophila* and mammals. dJub and hLIMD1 overexpression in wings and eyes resulted in a modest increase in size, as a result of increased cell number (Figures 1C, 1E, 1F, and 1R). In pupal eye epithelium, dJub localized to adherens junctions (AJs), predominantly interommatidial cells, colocalizing with DE-cadherin in a punctate pattern (Figures 1L–1O). The *HA-LIMD1* transgene also localized to AJs in wing larval disc epithelia (Figure S1I). This cellular localization of dJub is similar to that of mammalian Ajuba LIM proteins in mammalian epithelia [1].

We generated *djub* mutant alleles via FLP-FRT-based methods [12]. Two distinct yet overlapping deficiencies of the *djub* locus were generated (Figure S2A). Both deficiencies yielded identical results for all phenotypic studies. Flies hemizygous for each deficiency died at the late embryonic to first-instar larval stage. Female flies (heterozygous for $djub^{I}$ or $djub^{II}$) expressed 50% levels of dJub protein (Figure S2C). Ubiquitous expression of a WT *djub* transgene rescued lethality of both alleles, confirming that the loss of *djub*, and not flanking genetic material, was responsible and that *djub* is an essential gene.

When dJub was deleted in the eye by using *eyeless-FLP* (EGUF/hid) to produce eyes composed of over 90% $djub^{I}$ mutant cells [13], adult eyes were severely reduced in size (Figure 1Q). Mosaic analysis of $djub^{I}$ and WT twin-spot clones in eye and wing imaginal discs resulted in $djub^{I}$ mutant clones (Figures 1T and 1U, yellow arrows) that were significantly smaller than WT twin-spot clones (Figures 1S and 1U, red arrows). To verify that growth defects were specific to loss of *djub* function, we induced $djub^{I}$ clones throughout the wing imaginal disc while simultaneously expressing a WT mCherry-tagged *djub* transgene only in the posterior half of the wing disc via *engrailed-GAL4*. In the anterior compartment,

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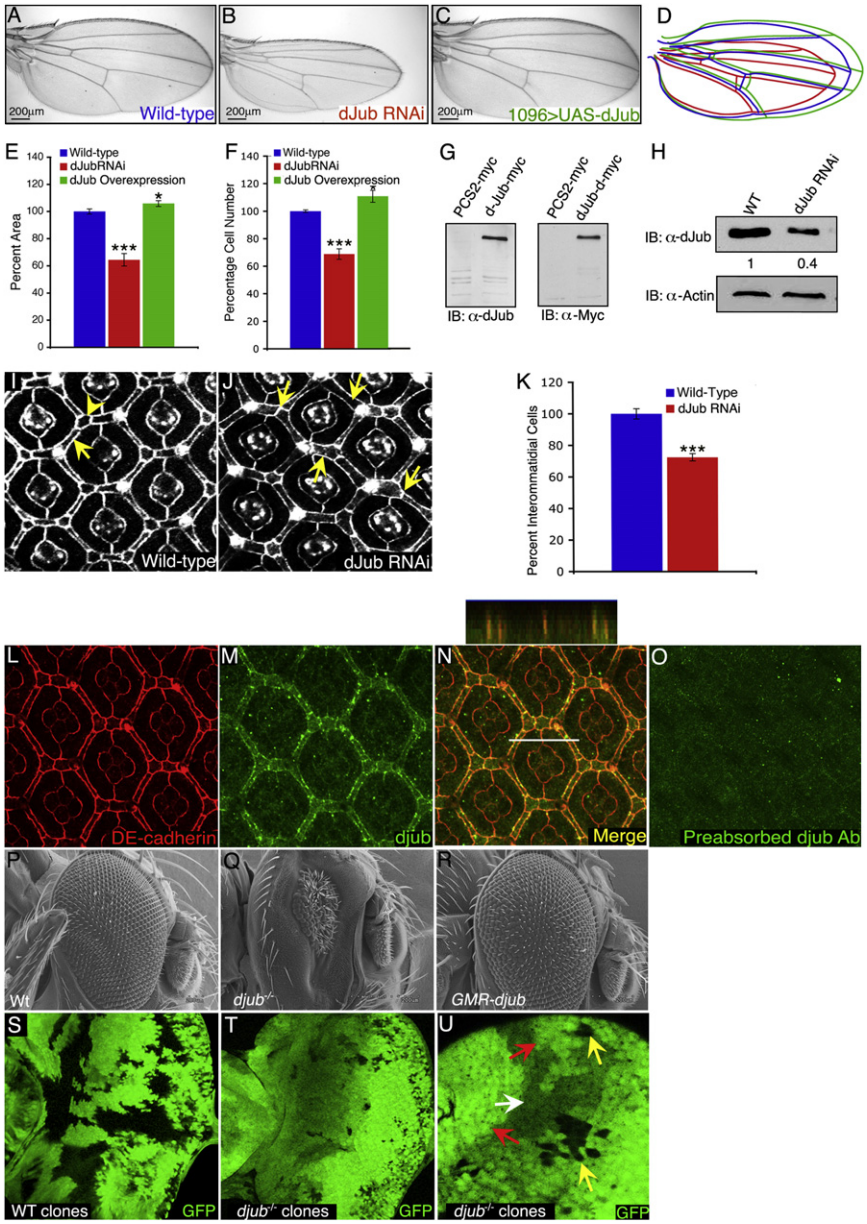

Figure 1. dJub Regulates Tissue Size by Controlling Cell Number

(A–C) Wings from wild-type (WT) female flies (A), female flies expressing dJub RNAi (B), or female flies expressing a *djub-mCherry* transgene (C). *1096-GAL4* was used to drive RNAi or transgene expression.

(D) Outlines of the wings in (A)–(C).

(E and F) Quantification of relative wing areas (E) and cell numbers (F) of genotypes in (A)–(C). Area and cell number measurements were taken from the wing region located between veins L4 and L5, and WT was defined as 100%.

(G) Extracts of mammalian HEK293 cells transfected with myc-dJub immunoblotted with dJub antiserum (left) or Myc antiserum (right).

(H) Immunoblot analysis of dJub protein levels in WT or dJub RNAi-expressing larval eye imaginal discs. Actin serves as loading control. Relative amount of dJub protein is indicated below each lane.

(I and J) Midpupal WT eyes (I) or *GMR-GAL4*-driven dJub RNAi-expressing eyes (J) stained for DE-cadherin. Secondary (arrows) and tertiary (arrowhead) interommatidial cells are highlighted in (I). Loss of interommatidial cells in dJub RNAi-expressing pupal eyes is denoted by arrows in (J).

(K) Quantification of relative numbers of interommatidial cells in WT versus dJub RNAi pupal eye. Interommatidial cells were counted in 20 fields, each containing a cluster of at least 7 ommatidia.

(L–N) Midpupal WT eyes stained for DE-cadherin (L) and dJub (M). Merged image is shown in (N). Z stack analysis of the line in (N) is shown above (N).

(O) Immunostaining with dJub antiserum preabsorbed with immunizing peptide.

(P–R) Scanning electron micrographs (SEMs) of female adult eyes.

(P) Wild-type.

(Q) *djub*^{l} generated via the EGUF/hid method, which results in eyes composed almost entirely of mutant tissue.

(R) *GMR-GAL4*-driven overexpression of a *UAS-djub-mCherry* transgene.

(S–U) Female third-instar larval eye imaginal discs containing WT (S) or *djub*^{l} mutant (T and U) clones marked by the absence of GFP expression (black).

(U) Enlarged view of *djub*^{l} and WT twin-spot clones. Yellow arrows identify *djub*^{l} clones, red arrows identify WT twin-spot clone containing two copies of Ubi-GFP, and white arrow identifies tissue carrying one copy of Ubi-GFP.

In all experiments, wings and eyes were dissected from female flies. In graphs, data are shown as mean percentages ± standard deviation, with n = 20 for each genotype. ***$p \leq 0.001$; *$p \leq 0.05$. Anterior is to the left for all larval imaginal discs.

djub^{l} clones were small and few in number (Figure S2E). In contrast, the posterior compartment contained more and larger clones, similar to WT clones induced in a WT background (Figures S2D–S2F).

djub Mutant Clones Exhibit Reduced Proliferation and Increased Apoptosis

The growth phenotype of *djub*^{l} null clones could result from decreased cell proliferation and/or increased apoptosis. In WT larval eye discs, undifferentiated cells lie anterior to the morphogenetic furrow and undergo asynchronous cell divisions (Figure S1J, white arrow). Posterior to the furrow, cells differentiate or undergo one more cell division, the second mitotic wave (Figure S1J, yellow arrow), after which they differentiate or die [14, 15]. Bromodeoxyuridine (BrdU) labeling of WT and *djub*^{l} eye discs, generated via the EGUF/hid method, revealed that *djub*^{l} eye discs displayed a strong reduction in cells undergoing asynchronous division anterior to the furrow (Figure S1J′, white arrow) and a near complete loss of the second mitotic wave (Figure S1J′, yellow arrow). During eye development, apoptosis determines the final number of cells in the eye [16]. Staining eye discs for activated caspase-3 revealed that *djub*^{l} eye discs contained increased number of caspase-3-positive cells (Figure S1K′). When the caspase inhibitor P35 was coexpressed with *djub*^{l}, the small-eye phenotype was partially rescued (Figure S3B). Relative to WT, *djub*^{l} clones exhibited decreased levels of *Drosophila* inhibitor of apoptosis 1 (DIAP1) (Figure 2A) and cyclin E (Figure 2C). dJub controlled transcription of DIAP1, because *djub*^{l} clones expressed less lacZ under control of the *diap1* gene

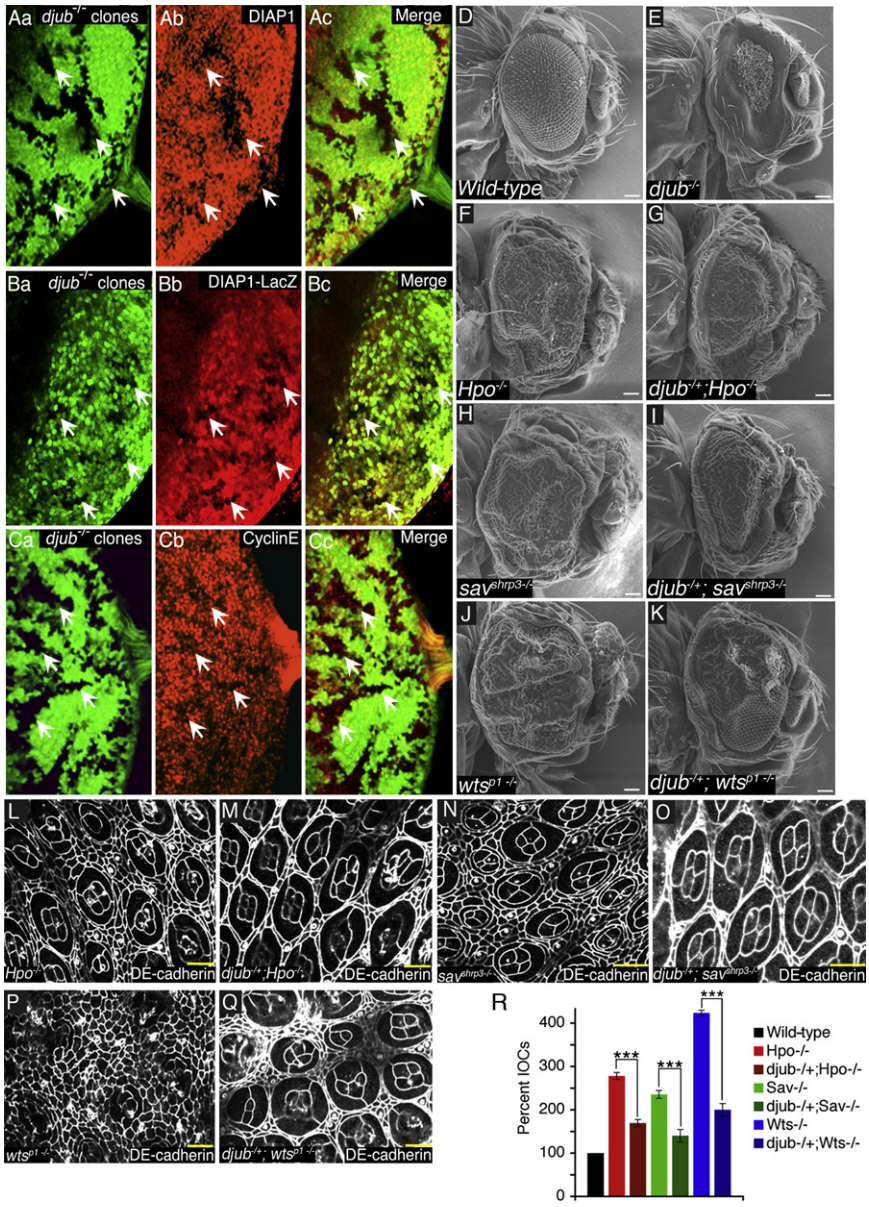

Figure 2. *djub* Affects Expression of DIAP1 and Cyclin E and Genetically Interacts with the Hippo Pathway

(A–C) Female third-instar larval eye imaginal discs containing *djub*^{1} clones (GFP-negative, white arrows) generated by *eyeless-FLP* (Aa–Ca) and stained for DIAP1 (Ab), DIAP1-lacZ (Bb), or cyclin E (Cb) expression. Merge images are shown at right (Ac–Cc). Anterior is to the left for all larval eye imaginal discs.

(D–K) SEMs of adult female *Drosophila* eyes of WT (D) and Hippo pathway mutants (F, H, and J) and *djub*^{1} (E) and Hippo pathway mutants containing a deletion of a single copy of *djub* (G, I, and K), as indicated. Scale bars represent 100 μm.

(L–Q) Midpupal eye dissection of Hippo pathway mutants (L, N, and P) or Hippo pathway mutants containing a deletion of a single copy of *djub* (M, O, and Q), as indicated, and stained for DE-cadherin to identify interommatidial cells. Scale bars represent 10 μm.

(R) Quantification of interommatidial cell numbers in 10 random fields containing 10 ommatidia each of the genotypes in (D) and (F)–(K). Data are shown as mean percentages ± standard deviation. ***$p \leq 0.001$.

promoter (Figure 2B). These data indicate that dJub regulates organ size by inhibiting apoptosis and promoting cell proliferation by influencing DIAP1 and cyclin E expression, respectively. *djub* deletion did not affect AJ organization, as determined by DE-cadherin staining of *djub*^{1} clones (Figure S3C).

DJub Genetically Interacts with the Hippo Pathway

The *djub* loss-of-function phenotype resembles that of *yorkie* (*yki*), which encodes a transcriptional coactivator, the activity of which is antagonized by the Hippo (Hpo) signaling pathway. Active Yki promotes proliferation and inhibits apoptosis by facilitating transcription of *cyclin E* and *diap1* [17, 18]. Activated Hpo kinase, in association with Salvador (Sav), a WW-repeat scaffolding protein, phosphorylates and activates Warts (Wts) kinase, which in association with Mob as tumor suppressor (Mats) phosphorylates Yki. Phosphorylation of Yki directs Yki to the cytoplasm, inhibiting its transcriptional regulation [8]. Given the similarity between *djub* and *yki* loss-of-function phenotypes, we hypothesized that dJub governs organ size either by affecting Yki activity directly or by affecting it indirectly by inhibiting Hippo pathway function. Hippo pathway mutants (*hpo*, *sav*, and *wts*) produce overgrown adult eyes (Figures 2F, 2H, and 2J) and pupal eyes with increased interommatidial cells (Figures 2L, 2N, 2P, 2R; Figures S3D–S3F) [19, 20]. Removing a copy of *djub* reduced the magnitude of *hpo* and *sav* phenotypes (Figures 2G, 2I, 2M, 2O, and 2R) and modestly affected the *wts* phenotype (Figures 2K, 2Q, and 2R). In a reciprocal manner, a 50% reduction in *wts* suppressed the dJub RNAi small-wing phenotype (Figures S4A–S4E), whereas a 50% reduction of *yki* enhanced this phenotype (Figures S4F–S4J). Taken together, these results suggest the possibility that *djub* and the Hippo pathway genetically interact. If so, then *djub* specifically interacts with the Hippo pathway, because no genetic interactions were observed between *djub* and *myc* or components of the insulin receptor signaling pathway, which is known to regulate organ size by affecting cell size (Figure S4K). dJub localization to AJs was unaltered in *wts*, *hpo*, and *sav* mutant pupal eyes (Figures S3D–S3F).

Epistatic Analysis Suggests that *djub* Acts Upstream of *wts* and *yki* but Downstream of *hpo*

To confirm that *djub* indeed genetically interacts with the Hippo pathway, we performed genetic epistasis experiments between *djub* and *yki*, *wts*, and *hpo*. Mosaic analysis with a repressible cell marker (MARCM) pupal eye clones of *djub*^{1} alone result in small clones (Figure 3B, quantified in Figure 3I; Figure S2H), whereas MARCM clones overexpressing Yki or

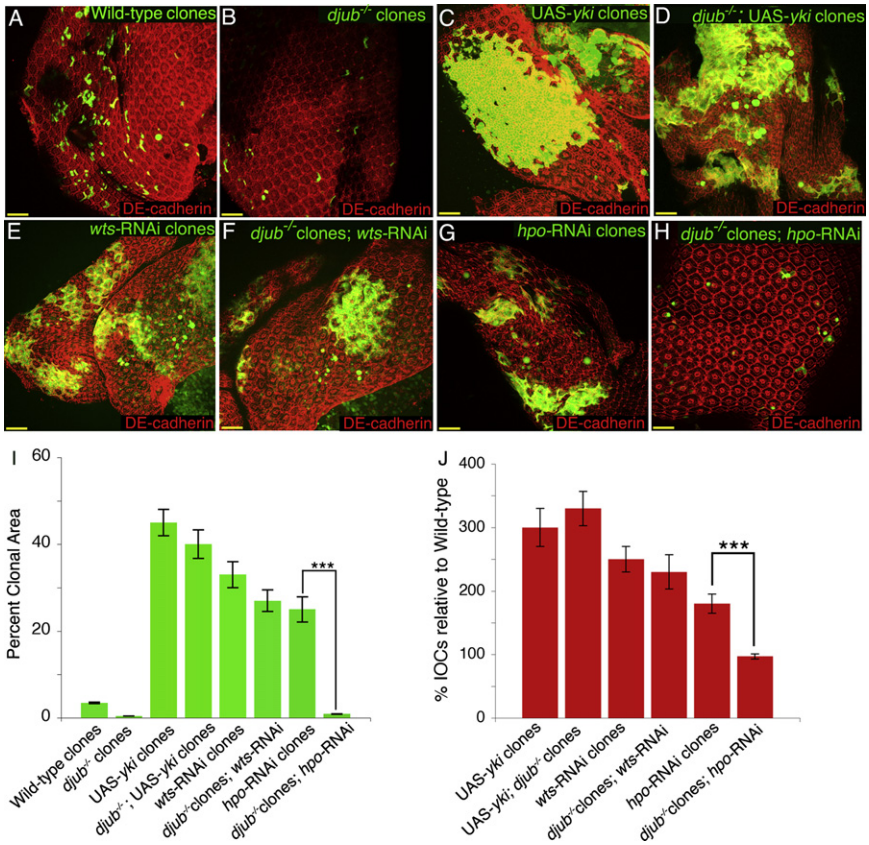

Figure 3. *djub* Is Epistatic to *hpo* Based on Clonal Area and Interommatidial Cell Numbers

(A–H) Female midpupal eyes stained for DE-cadherin (red). Scale bars represent 20 μm.

(A) WT, GFP-positive MARCM clones.

(B) *djub*^{1} MARCM clones (GFP-positive).

(C) MARCM clones overexpressing Yorkie (Yki) (GFP-positive).

(D) MARCM clones mutant for *djub*^{1} and overexpressing Yki (GFP-positive).

(E) MARCM clones expressing *wts* RNAi (GFP-positive).

(F) MARCM clones mutant for *djub*^{1} and expressing *wts* RNAi (GFP-positive).

(G) MARCM clones expressing *hpo* RNAi (GFP-positive).

(H) MARCM clones mutant for *djub*^{1} and expressing *hpo* RNAi (GFP-positive).

(I) Quantification of the clonal area (GFP-positive) for each genotype as a percentage of the entire pupal eye area.

(J) Quantification of the percent increase of interommatidial cells within the clonal area (GFP-positive) as compared to wild-type (set at 100% interommatidial cells [IOCs]) for each genotype.

Data in (I) and (J) are shown as mean percentages ± standard deviation, with n = 10 for each genotype. ***$p \leq 0.001$.

depleted of Wts or Hpo exhibit increased clonal area (Figures 3C, 3E, and 3G, quantified in Figure 3I) as well as overproliferation of interommatidial cells [17, 21–23] (Figures S2I, S2K, and S2M, quantified in Figure 3J). *djub*^{1} mutant MARCM clones overexpressing *yki* displayed a phenotype identical to overexpression of *yki* alone (Figure 3D, quantified in Figure 3I; Figure S2J, quantified in Figure 3J). *djub*^{1} mutant MARCM clones depleted of *wts* resembled *wts* RNAi clones (Figure 3F, quantified in Figure 3I; Figure S2L, quantified in Figure 3J); however, removing *djub* in *hpo* RNAi MARCM clones resulted in a *djub*^{1}-like phenotype (Figure 3H, quantified in Figure 3I; Figure S2N, quantified in Figure 3J). This epistatic analysis suggests that *djub* acts downstream of *hpo* but upstream of *wts* and *yki*, but because the core Hippo pathway proteins (Hpo, Sav, Wts, and Mats) are thought to function as a complex, a precise epistatic relationship is difficult to conclude.

Ajuba LIM Proteins/dJub Associate with LATS/Wts and WW45/Sav in Mammalian and *Drosophila* Cells and Influence YAP Activity in Mammalian Cells

The Hippo pathway is highly conserved between *Drosophila* and vertebrates [5, 8, 23, 24], and human LIMD1 rescues the cell growth defects of dJub-depleted *Drosophila* wings (Figures S1G and S1H). To determine whether Ajuba LIM proteins physically and functionally interact with Hippo pathway components in cells, we tested whether the mammalian homologs of dJub (Ajuba, LIMD1, and WTIP) associated with (i.e., coimmunoprecipitated) mammalian orthologs of Hippo pathway members in human epithelial cells. All three Ajuba subfamily members associated strongly with LATS1/2, and Ajuba and WTIP associated with WW45, but none associated with MST1/2 or YAP (Figures 4A–4C). The interaction between LATS and WW45 and Ajuba family proteins was specific, because zyxin, a closely related LIM protein, failed to associate with either LATS or WW45 (Figure 4D). In *Drosophila* S2 cells, dJub associated with Wts and Sav but not Hpo (Figure S4L). A weak association between dJub and Yki was noted, but this was >10-fold less than that observed for Wts and Sav and may be nonspecific, because transfected Yki was massively overexpressed in S2 cells (Figure S4L). To determine whether these protein interactions were functionally relevant, we asked whether Ajuba LIM proteins affected YAP phosphorylation [8, 18, 25]. In cells transfected with MST1, WW45, or LATS1/2 alone, there was variable increase in phospho-YAP levels; however, when cotransfected with LIMD1, phospho-YAP levels were decreased in all instances (Figure 4E). Overexpression of dJub in *Drosophila* imaginal discs did not change the level or subcellular localization pattern of Yki or other Hippo pathway targets, namely Ex and Diap1. This may be because only a small (10%) increase in wing size occurs in wings overexpressing dJub (Figures 1C, 1E, and 1F). In another approach, Ajuba and LIMD1 were RNAi-depleted in MDCK cells, and phospho-YAP levels were determined in cells at differing densities [7]. Analysis of MDCK cells depleted of all three Ajuba LIM proteins was not possible because these cells died, like *Drosophila* cells lacking dJub. Compared to control MDCK cells, in cells depleted of Ajuba and LIMD1, basal phospho-YAP levels were increased 2.5-fold at all densities (Figure 4F). These results demonstrate that mammalian Ajuba LIM proteins/dJub specifically associate with LATS/Wts and WW45/Sav in cells, and that in mammalian cells, these associations antagonize the phosphorylation of YAP.

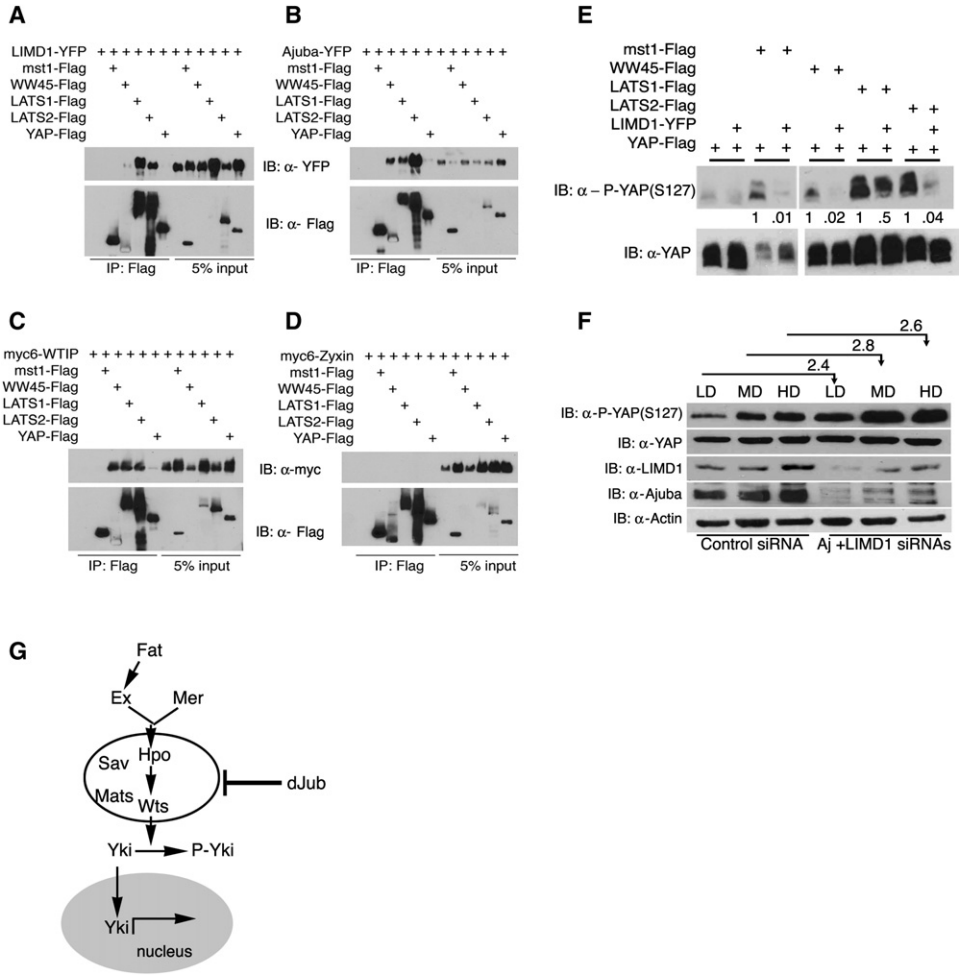

Figure 4. Ajuba LIM Proteins Associate with Components of the Hippo Pathway in Mammalian Cells and Influence YAP Phosphorylation

(A–D) HEK293 cells were cotransfected with LIMD1-YFP (A), Ajuba-YFP (B), Myc-WTIP (C), or Myc-zyxin (D) and Flag-tagged Mst1, LATS1/2, WW45, or YAP, as indicated. Cell lysates were immunoprecipitated for each Hippo pathway member (anti-Flag), and bound products were immunoblotted (IB) for the presence of each LIM protein (anti-YFP or anti-Myc). Immunoblots of input controls (5%) are shown at the right of each panel.

(E) HEK293 cells were transfected with the indicated member of the Hippo pathway in the absence or presence of LIMD1-YFP. Levels of phospho-S127-YAP (upper panel) or total YAP (lower panel) were then determined by immunoblot analysis. Relative amounts of phospho-S127-YAP with respect to total YAP protein are indicated below each lane.

(F) MDCK cells were transfected with control Luc siRNA (lanes 1–3) or Ajuba and LIMD1 siRNAs (lanes 4–6) and then plated at low (LD), medium (MD), and high density (HD). Amount of S127-YAP phosphorylation relative to total YAP for each density within control and Ajuba/LIMD1-depleted cells was determined by immunoblotting. The relative level of YAP phosphorylation for each density between control and Ajuba/LIMD1-depleted cells is indicated above the lanes.

(G) Working model, based upon results herein, for how Ajuba LIM proteins could influence Hippo pathway signaling.

Our work indicates that the Ajuba LIM proteins/dJub influence organ growth through negative regulation of the Hippo signaling pathway in flies, and likely mammals. Genetic and biochemical data suggest that Ajuba LIM proteins/dJub likely interface with the Hippo pathway at the level of LATS/Wts. Prior work has described an interaction between Ajuba and LATS at centrosomes that influences mitotic centrosome and spindle organization [26]. Whether this contributes to the *djub*^{1} phenotype, we cannot exclude, because mitotic damage can lead to apoptotic cell death.

Precisely how Ajuba LIM proteins/dJub influence LATS/Wts-mediated inactivation of YAP/Yki remains to be determined, but possibilities include inhibition of LATS/Wts activation by upstream kinases (MST/Hpo), inhibition of the ability of LATS/Wts to phosphorylate YAP/Yki, or modulation of the subcellular localization of LATS/Wts or WW45/Sav and thus their access to the Hippo pathway. The regulatory relationship between Ajuba LIM proteins/dJub and LATS/Wts may not be simply unidirectional, because LATS has been shown to phosphorylate Ajuba [26]. Ajuba LIM proteins are components of AJs. Upstream members of the Hippo pathway, including atypical cadherins (Fat and Dachsous), Expanded, and Merlin, also localize to AJs, leading to the hypothesis that AJs are nodal points for initiation and/or regulation of Hippo signaling [25, 27–32]. How these upstream components activate MST/Hpo kinase is unknown. The Hippo pathway is thought to regulate cell contact growth inhibition [7]. In subconfluent noncontacted cells, Ajuba LIM proteins are cytosolic while YAP is nuclear, and cells proliferate [7]. When cells achieve confluence, Ajuba proteins are recruited to AJs while YAP is phosphorylated and relocalized to the cytosol, and cell proliferation ceases. Whether these events are related is not known, but the

finding that Ajuba proteins associate with and inhibit LATS/Wts-mediated phosphorylation of YAP raises the possibility that the recruitment of Ajuba LIM proteins/dJub to AJs in confluent cell cultures may "release" LATS/Wts, allowing for Hippo pathway-mediated YAP/Yki phosphorylation, inactivation, and growth arrest.

Supplemental Information

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2010.02.035.

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